SURVEY OF HUMAN MILK FOR VOLATILE *N*-NITROSAMINES AND THE INFLUENCE OF DIET ON THEIR FORMATION

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Abstract—Human milk was analysed for volatile *N*-nitrosamines and the influence of diet on their possible presence and formation was assessed. 175 samples were obtained from 16 different nursing women. Analysis of samples collected at random, to ascertain baseline levels, indicated that 76.5% of the human milk samples contained less than 0.2 ppb *N*-nitrosodimethylamine (minimum level of reliable measurement). No other volatile nitrosamines were detected. Expressed milk was collected from volunteers over a 6-hr period after eating meals which included bacon (a source of preformed nitrosamines) and at times a vegetable high in nitrate. Eating a meal containing bacon did not result in increased nitrosamine levels in milk. In certain individuals, eating a meal of bacon and a vegetable high in nitrate occasionally resulted in higher levels of *N*-nitrosodimethylamine in their milk.

INTRODUCTION

Volatile *N*-nitrosamines (NAs) have been found in many different foods (Gray, 1981). They have also been detected in human blood (Fine, Ross, Rounbehler *et al.* 1977; Gough, Webb & Swann, 1983; Lakritz, Simenhoff, Dunn & Fiddler, 1980; Yamamoto, Yamada & Tanimura, 1980) and urine (Brooks, Cherry, Thacker & Alley, 1972; Hicks, Gough & Walters, 1978). The presence of NAs in humans may be a direct result of the ingestion of the preformed NAs or may be due to endogenous formation from NA precursors. *In vivo* formation in man was demonstrated by the appearance of *N*-nitrosodiphenylamine (NDPA) following ingestion of nitrate and diphenylamine in an individual diagnosed as achlorhydric (Sander & Seif, 1969). Recently Ohshima & Bartsch (1981) demonstrated the formation and subsequent excretion of *N*-nitrosoproline (NPRO) in the urine of a healthy subject following ingestion of proline and a vegetable high in nitrate (beet juice).

Extensive studies with pharmaceuticals have elucidated the mechanism for transport of drugs, pesticides, and other compounds from plasma to milk across the mammary membrane (Knowles, 1965). Laboratory studies conducted on lactating animals have shown that administered NAs can translocate into the milk. Mohr & Althoff (1971) injected *N*-nitrosodiethylamine (NDEA) sc into nursing hamsters resulting in the death of one third of their suckling offspring. Administration of ^{14}C-labelled NDEA ip to nursing hamsters resulted in the appearance of the labelled NA in the blood and milk within 5 and 45 min, respectively (Spielhoff, Bresch, Hönig & Mohr, 1974). The introduction of NAs directly into the stomachs of rats (Schoental, Gough & Webb, 1974) or goats (Juskiewicz & Kowalski, 1974) by gastric intubation led to detectable levels of NDEA and *N*-nitrosodimethylamine (NDMA) in the milk within 2 hr and NDPA appeared even more rapidly. It is reasonable to assume that similar processes can occur in humans. Melikian, La Voie, Hoffmann & Wynder (1981) analysed blood and breast fluid from nonlactating women for volatile NAs before and after the ingestion of a meal high in nitrate. NAs were not detected in the breast fluid. The purpose of this study was to determine whether NAs were normally present in human milk, and whether there was a correlation between the NA level and the diet.

EXPERIMENTAL

Sixteen nursing women 2 wk–9 months *post partum* participated in this investigation. The women maintained a record of all food eaten from 14 hr before initiation of the study until all their milk samples had been collected. All studies were started in the morning. The initial sample (baseline) was obtained by collecting the expressed milk that had accumulated overnight. Depending upon the nature of the study, the subjects were instructed to eat either a specified diet (which excluded juices rich in vitamin C) or to follow their normal dietary habits. Samples were collected 1, 2, 3 and 6 hr after initiation of the study, unless otherwise instructed. Samples (30 ml) were collected into glass bottles containing 1 ml of 0.6 N-KOH, mixed, and immediately frozen.

Artefact formation. It was not feasible to collect and analyse the human milk samples for NA on the same day. Therefore, before beginning this investigation, a series of studies was performed to determine the best method of storage to prevent

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Abbreviations: DCM = dichloromethane; GC–TEA = gas chromatography–thermal energy analysis; NA = nitrosamine; NaAsc = sodium ascorbate; NDEA = *N*-nitrosodiethylamine; NDMA = *N*-nitrosodimethylamine; NDPA = *N*-nitrosodiphenylamine; NPRO = *N*-nitrosoproline; TEA = Thermal Energy Analyzer.

artefactual NA formation. Milk samples, obtained 6 hr after expression, were either made alkaline with 0.6 N-KOH or treated with 1000 ppm sodium ascorbate (NaAsc). These samples were divided. A portion of each was stored in the refrigerator overnight and assayed, or frozen for 1 wk and then assayed. The results from this storage study were compared with those of nontreated controls. Artefactually formed NAs were not detected using either method of storage. We chose to treat the sample with KOH rather than NaAsc prior to storage, since NaAsc may be rapidly oxidized. To assess the effect of excess NO_2^- on possible NA formation in milk stored over base and frozen, 1 ppm $NaNO_2$ was added; NAs were not detected.

N-Nitrosamine analysis. Milk samples were analysed for volatile nitrosamines by the procedure described by Lakritz & Pensabene (1981). *N*-Nitrosomethylethylamine (1.0 ml of a 0.05 μg/ml solution) was added to each sample as an internal standard. All reagents were checked before analysis for traces of NAs. Samples (25 g) were added to flasks containing 50 ml 5 N-NaOH, 8 g $Ba(OH)_2$ and 25 ml water, and the mixture was distilled. NaCl (15 g) was added to the aqueous distillate, which was then extracted three times with 100 ml dichloromethane (DCM). The DCM layer was washed with 25 ml 6 N-HCl and with 25 ml 5 N-NaOH, dried over anhydrous Na_2SO_4, and concentrated to 1.0 ml in a Kuderna-Danish evaporator flask equipped with a 4-ml concentrator tube.

The nitrosamines were detected and quantitated using a Varian-Aerograph Model 1720 gas chromatograph (Palo Alto, CA) inferfaced with a Thermal Energy Analyzer (TEA; Model 502 Thermo Electron Corp., Waltham, MA). The GC was equipped with a 9′ × 1/8″ stainless-steel column packed with 15% Carbowax 20M TPA on 60–80 mesh Gas Chrom P. The injector port temperature was 180°C and the column temperature was programmed from 110 to 220°C at 4°C/min, with a helium flow rate of 42 ml/min. NDMA, NDEA and NPYR eluted at 4.2, 5.6 and 14.5 min, respectively. The TEA was operated under conditions similar to those used by Fine & Rounbehler (1975). Samples containing concentrations as low as 0.1 ppb could be detected confidently. Presumptive confirmation was obtained by the UV photolysis procedure described by Doerr & Fiddler (1977).

Detection limit. Using this method, the minimum detectable level of NDMA on this GC–TEA system was 0.1 ppb. Analysis of variance of six samples analysed in duplicate indicate that the standard deviation due to repeatability of NDMA determinations was 0.24 ppb (CV 12.41%; 0.27 ppb uncorrected) and the standard deviation due to repeatability of recovery of the NMEA internal standard was 3.49% (CV 3.56%).

Nitrite analyses. Milk and food samples were analysed for nitrite using chemiluminescence detection techniques (Doerr, Fox, Lakritz & Fiddler, 1981). A 2-ml sample was injected into a sealed three-neck 100-ml round-bottomed flask which had been previously flushed with He to remove air, and which contained approximately 50 mg each of NaAsc and tartaric acid. The sample was stirred and the nitric oxide, liberated from the acidified sample, was introduced via a gas-sampling valve into the chemiluminescence detector for quantitative analysis. The chemiluminescence detector used for the NO_2^- analysis is part of the same detection system incorporated in the TEA and used for the analysis of NAs.

Table 1. *N*-Nitrosodimethylamine in human milk

NDMA (ppb*)	No. of samples
ND	35
>0.1	4
>0.2	5
>0.3	3
>0.4	2
>0.6	1
> 1.0	1 (1.1 ppb)

ND = Not detected (<0.1 ppb).
*ppb ≅ ng/ml.
Samples were obtained from 13 subjects.

RESULTS AND DISCUSSION

To determine whether NAs are "normal" constituents of human milk, 51 samples were obtained from 13 nursing women. These volunteers were under no dietary restrictions, they ate their customary foods, and collected samples at will. NDMA was the only volatile NA detected. NAs were not detected in 69% of the samples analysed (Table 1). Considering the response on the TEA detector to NDMA, the nature of the samples, and method repeatability, only values greater than 0.2 ppb were considered meaningful. NDMA in concentrations greater than 0.2 ppb was found in 23.5% of samples.

The composition of human milk is influenced by several factors including: number of days postparturition, age of mother, season of the year, and number of siblings (Blanc, 1981). The effects due to these factors are essentially constant for an individual over a short period of time and can be ignored. Other variables, such as the time of collection (a.m. or p.m.), and whether the milk was "fore" milk (expressed at the start of the feed) or "hind" milk (collected towards the end of a feed) have considerable influence on the composition of milk. For example, the concentration of proteins and lipids are approximately 1.3 and 3 times greater in hind than in fore milk, and may influence the transport of NAs across the cell membrane. To determine the effect due to compositional differences between fore and hind milk on NA levels, five samples of both fore and hind milk from three subjects were collected over several weeks. Fore milk was collected from an engorged breast before feeding the baby and hind milk from the almost depleted breast immediately after feeding. No substantial differences between fore and hind milk were noted. NDMA was not detected in any of the fore or hind milk samples from two subjects. It was detected at 0.1 ppb in one fore and two hind milk samples and at 0.2 ppb in two fore and one hind milk sample from the third subject. In subsequent experiments, samples were collected without regard for this parameter. All studies started in the morning and

Table 2. Effect of a meal containing bacon on *N*-nitrosodimethylamine in human milk

	Concn of *N*-nitrosodimethylamine (ppb*)				
		Time after test meal (hr)			
Subject	Baseline†	1	2	3	6
1	ND	ND	ND	ND	ND
1	0.1	0.1	ND	0.2	ND
1	ND	ND	0.1		
2	ND	ND	ND	ND	0.1
3	0.1	ND	0.1	0.1	0.1
4	ND	0.2	ND	0.3	0.1
5	1.1	0.8	1.3	ND	0.2
5	0.1	0.6	0.2	0.2	0.2
6	0.2	ND	0.1	0.2	0.2

ND = Not detected (<0.1 ppb)

*ppb ≅ ng/ml.

†Samples collected in morning before eating test meal.

ended approximately 6 hr later to minimize diurnal variations.

Studies with humans have demonstrated *in vivo* formation of NAs after ingestion of precursor amines and NO_3^- (Ohshima & Bartsch, 1981; Sander & Seif, 1969); studies on lactating animals have shown that following the administration of NAs these compounds, or their metabolites, can translocate into the milk (Mohr & Althoff, 1971; Spielhoff *et al.* 1974). The effect of ingesting preformed NAs on NA levels in human milk was tested in seven volunteers who included bacon (approximately six strips) with their normal breakfast (Table 2). Eating bacon exerted no apparent influence on the NA level in human milk collected over a 6-hr period, even though commercially processed bacon often contains 2–6 ppb *N*-nitrosopyrrolidine and 3–5 ppb NDMA. Milk samples collected from a single subject (no. 5) contained 0.8 and 1.3 ppb NDMA at 1 and 2 hr, respectively. Since the baseline sample of this subject contained 1.1 ppb NDMA, the presence of this nitrosamine in the milk after initiation of the study cannot be solely attributed to the bacon. Another series of milk samples was collected from the same subject. The results showed 0.6 ppb NDMA after 1 hr.

The influence of nitrate in the diet on *in vivo* NA formation was also tested. Four volunteers ate 8 oz of either spinach or red beet and bacon with their breakfast. It has been postulated that 5% of the ingested NO_3^- is reduced by saliva to NO_2^- (Spiegelhalder, Eisenbrand & Preussman, 1976). Urinary excretion of NPRO was found to be proportional to the amount of amine ingested and increased exponentially as the amount of NO_3^- ingested increases (Oshima & Bartsch, 1981). Analysis of the red beet and spinach samples indicated that they contained 1400–1800 ppm NO_3^- and 1 ppm NO_2^-. All milk samples from two of the four volunteers were essentially devoid of any NA (Table 3). Several samples of the milk obtained from the two other subjects (Nos 2 & 3) contained NDMA at concentrations of 1 ppb and greater. These NDMA-positive samples were photolysed (Doerr & Fiddler, 1977) and reassayed for NDMA. Its disappearance strongly suggested that the peaks were indeed NDMA. Mass spectral analysis of the sample containing 17 ppb was attempted; however, even after column chromatographic clean-up, the presence of an interfering peak prevented absolute confirmation.

It was not possible to conclude that eating a combination of vegetables containing high levels of NO_3^- with bacon, resulted in a subsequent increase in NDMA levels in milk. The presence of 1 ppb or more NDMA in three out of four samples from subject no. 2 could indicate that this individual may be predisposed to form NDMA, or perhaps she was unable to metabolize NDMA, as readily or rapidly as the others. Both this subject, and no. 3, began their respective studies with above average NDMA levels in their baseline samples.

Additional diet studies with a larger population, including analysis of serial blood samples, are needed to assess definitively the possible role of preformed NA and NA precursors in the formation of volatile NAs in human milk.

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Table 3. Effect of ingesting a meal containing red beet or spinach, and bacon on volatile nitrosamine content of human milk

		Concn of *N*-nitrosodimethylamine (ppb*)				
			Time after test meal (hr)			
Subject	Vegetable	Baseline†	1	2	3	6
1	Red beet	ND	ND	ND	ND	ND
1	Red beet	ND	ND	0.1	ND	ND
2	Red beet	0.6	0.3	0.1	0.4	0.3
2	Red beet	0.3	17.1‡	0.3	0.2	1.0
2	Red beet	0.2	ND	1.3‡	ND	ND
3	Red beet	0.4	0.2	0.2	0.4	3.2
1	Spinach	ND	ND	ND	ND	0.1
1	Spinach	ND	ND	ND	ND	ND
2	Spinach	0.3	0.2	1.0‡	0.1	ND
8	Spinach	ND	0.1	ND	0.1	ND

ND = Not detected (<0.1 ppb)

*ppb ≅ ng/ml.

†Sample collected in morning before eating test meal.

‡Sample photolysed and positive for NA.

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